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PATENT- OG VAREMÆRKESTYRELSEN

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Modtaget

SCREENING METHOD

FIELD OF THE INVENTION

The application relates to a method of screening lipolytic enzymes to identify a candidate for use as a baking additive which can improve the properties of a baked product when added to a dough.

BACKGROUND OF THE INVENTION

It is known that various properties of a baked product can be improved by adding a lipolytic enzyme. The prior art provides a large number of lipolytic enzymes obtained from natural sources or by protein engineering. Evaluation in full-scale baking tests generally require a major effort for isolating and producing each enzyme in sufficient quantity, so screening methods are useful to select candidates for full-scale testing. WO 0032758 discloses a method of screening lipolytic enzymes for use in baking based on their activity towards ester bonds in short-chain and long-chain triglycerides, digalactosyl diglyceride and a phospholipid, particularly phosphatidyl choline (lecithin).

The lipids present in wheat flour are known to consist mainly of triglycerides, phospholipids and galactolipids. It is known that the phospholipids in wheat flour consist mainly of lyso phosphatidyl choline and phosphatidyl choline, but also include N-acyl phosphatidyl ethanolamine (APE) and N-acyl lysophosphatidyl ethanolamine (ALPE).

SUMMARY OF THE INVENTION

The inventors have developed a method of screening lipolytic enzymes to identify candidates for a baking additive which can improve the properties of a baked product when added to the dough. The improved properties may include a larger loaf volume, an improved shape factor, an improved crumb structure and/or improved dough stability e.g. improved tolerance towards extended proofing.

Accordingly, the invention provides a method of screening a lipolytic enzyme for use as a baking additive, comprising:

a) contacting the enzyme with N-acyl phosphatidyl ethanolamine (APE) or N-acyl lysophosphatidyl ethanolamine (ALPE), and

b) detecting hydrolysis of an ester bond in the APE or ALPE.

DETAILED DESCRIPTION OF THE INVENTION

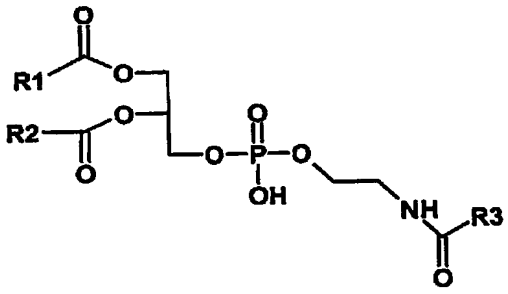
Lipolytic enzymes

The method of the invention is applicable to screening of lipolytic enzymes. The lipolytic enzymes to be tested may be chosen among the large number of lipolytic enzymes known in the prior art, e.g. those described in WO 0032758. The enzymes to be tested may include naturally occurring enzymes, particularly from microorganisms such as fungi and bacteria, as well as variants made by protein engineering, e.g. those described in WO 0032758.

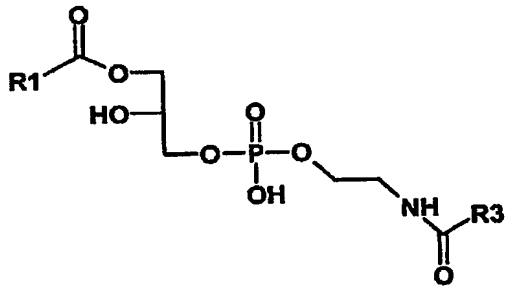
The lipolytic enzymes may be tested in crude or isolated form. In particular, it may be of interest to purify the enzymes sufficiently to allow determination the amount of enzyme protein.

APE or ALPE

The method of the invention uses a substrate which is N-acyl phosphatidyl ethanolamine (APE) or N-acyl lysophosphatidyl ethanolamine (ALPE) having the following structures, where ALPE may have the R_1-CO attached to the sn-1 or the sn-2 position of the lysophosphatidyl group. R_1-CO, R_2-CO and R_3-CO are each a fatty acyl, particularly an unsubstituted saturated straight-chain fatty acyl group having 12-22 carbon atoms, e.g. palmitoyl (C16:0), stearoyl (C18:0), oleoyl (C18:1) or linoleoyl (C18:2).



APE



ALPE

APE and ALPE for use in the screening method may be isolated from wheat flour or may be synthesized, e.g. as described in the examples.

Contact and hydrolysis

The lipolytic enzyme activity of interest acts to hydrolyze an ester bond in APE or ALPE. Thus, when using APE as the test substrate, it liberates one or two fatty acids (R_1-COOH and/or R_2-COOH) to form ALPE or N-acyl L-alpha-glyceryl-phosphoryl-ethanolamine (N-GPE). When using ALPE as the test substrate, the activity of interest hydrolyzes ALPE into the free fatty acid R_1-COOH and N-acyl L-alpha-glyceryl-phosphoryl-ethanolamine (N-GPE). It

may be of interest to test a number of lipolytic enzymes on the basis of equal amount of enzyme protein.

The contacting and testing of the lipolytic enzymes can conveniently be done as a plate assay, by thin-layer chromatography (TLC) or by high-performance liquid chromatography (HPLC), e.g. as described in the examples.

Screening system

According to the invention, the lipolytic enzymes are tested for their hydrolytic activity on APE or ALPE. This may be combined with testing of the hydrolytic activity on other substrates and to use the combined results to select candidates for further testing, e.g. using the test substrates described in WO 0032758. Thus, lipolytic enzymes may be chosen having a high hydrolytic activity on ester bonds in a C_{16}-C_{20} triglyceride, digalactosyl diglyceride and/or phosphatidyl choline (lecithin). The lipolytic enzyme may be chosen to have low activity on ester bonds in a C_4-C_8 triglyceride, a monoglyceride, digalactosyl monoglyceride and/or lysophosphatidyl choline (lysolecithin).

Use of screening results

Based on the hydrolytic activity towards APE/ALPE and optionally other substrates, a candidate may be selected and may be tested further by adding it to a dough and baking the dough to make a baked product. The enzyme may be added at a dosage of 0.1-10 mg enzyme protein per kg of flour, e.g. about 1 mg/kg. This may be evaluated by determining properties such as loaf volume, shape factor, crumb structure and/or dough stability e.g. tolerance towards extended proofing by conventional methods, e.g. as described in WO 0032758.

The lipolytic enzymes selected through the screening method of the invention may be added to the dough singly or in combination, e.g. as described in WO 02/03805. Optionally, an additional enzyme may also be added to the dough. The additional enzyme may be another lipolytic enzyme, an amylase, particularly an anti-staling amylase, an amyloglucosidase, a cyclodextrin glucanotransferase, or the additional enzyme may be a peptidase, in particular an exopeptidase, a transglutaminase, a cellulase, a hemicellulase, in particular a pentosanase such as xylanase, a protease, a protein disulfide isomerase, e.g., a protein disulfide isomerase as disclosed in WO 95/00636, a glycosyltransferase, a branching enzyme (1,4-α-glucan branching enzyme), a 4-α-glucanotransferase (dextrin glycosyltransferase), a lactase (galactosidase), or an oxidoreductase, e.g., a peroxidase, a laccase, a glucose oxidase, a pyranose oxidase, a lipoxygenase, an L-amino acid oxidase or a carbohydrate oxidase.

The amylase may be a fungal or bacterial alpha-amylase, e.g. from *Bacillus*, particularly *B. licheniformis* or *B. amyloliquefaciens*, or from *Aspergillus*, particularly *A. oryzae*, a beta-amylase, e.g. from plant (e.g. soy bean) or from microbial sources (e.g. *Bacillus*). The amylase

may be an anti-staling amylase, as described in WO 99/53769, i.e. an amylase that is effective in retarding the staling (crumb firming) of baked products, particularly a maltogenic alpha-amylase, e.g. from *Bacillus stearothermophilus* strain NCIB 11837.

Dough

The dough generally comprises wheat meal or wheat flour and/or other types of meal, flour or starch such as corn flour, corn starch, rye meal, rye flour, oat flour, oat meal, soy flour, sorghum meal, sorghum flour, potato meal, potato flour or potato starch.

The dough may be fresh, frozen or par-baked.

The dough is normally a leavened dough or a dough to be subjected to leavening. The dough may be leavened in various ways, such as by adding chemical leavening agents, e.g., sodium bicarbonate or by adding a leaven (fermenting dough), but it is preferred to leaven the dough by adding a suitable yeast culture, such as a culture of *Saccharomyces cerevisiae* (baker's yeast), e.g. a commercially available strain of *S. cerevisiae*.

The dough may also comprise other conventional dough ingredients, e.g.: proteins, such as milk powder, gluten, and soy; eggs (either whole eggs, egg yolks or egg whites); an oxidant such as ascorbic acid, potassium bromate, potassium iodate, azodicarbonamide (ADA) or ammonium persulfate; an amino acid such as L-cysteine; a sugar; a salt such as sodium chloride, calcium acetate, sodium sulfate or calcium sulfate.

The dough may comprise fat (triglyceride) such as granulated fat or shortening, but the invention is particularly applicable to a dough where less than 1 % by weight of fat (triglyceride) is added, and particularly to a dough which is made without addition of fat.

The dough may further comprise an emulsifier such as mono- or diglycerides, diacetyl tartaric acid esters of mono- or diglycerides, sugar esters of fatty acids, polyglycerol esters of fatty acids, lactic acid esters of monoglycerides, acetic acid esters of monoglycerides, polyoxyethylene stearates, or lysolecithin, but the invention is particularly applicable to a dough which is made without addition of emulsifiers (other than optionally phospholipid).

EXAMPLES

Example 1: Preparation of substrates

1.1 Isolation of APE and ALPE from wheat flour

Wheat flour (1 kg) was extracted twice with MeOH (1.5 L, stirring for 30 min). The extracts were concentrated and the residue re-dissolved in hexane (1 L) and concentrated. Yield of lipid extract: 8.5 g. The lipid extract was applied to a column packed with silica gel (120 g), which was preconditioned with 1 L of hexane/2-propanol/butanol/H_2O (60:30:7:3). Neutral lipids and carotenoids were removed by elution with hexane (800 mL) and then EtOAc (1.2 L).

Galactolipids were removed by eluting with toluene/acetone (1:1, 800 mL, MGDG) and acetone (9 L, DGDG). Finally, phospholipids (~1.1 g) could be eluted with MeOH (1 L). The individual phospholipids could be isolated by flash chromatography ($CHCl_3$/MeOH/H_2O: 65:25:4) to give pure fractions of APE and ALPE. The structures were verified by ^1H NMR and MS analysis.

1.2 Synthesis of ALPE

^1H NMR spectra were recorded on a Varian Mercury 400 MHz at 30°C. Flash chromatography was accomplished using a FLASH 40i chromatography module from Biotage. 1-Oleoyl-2-hydroxy-sn-glycero-3-phosphoethanolamine was purchased from Avanti. Linoleic anhydride was purchased from Sigma. All solvents were purchased from Merck.

N-Linoleoyl-1-oleoyl-2-hydroxy-sn-glycero-3-phosphoethanolamide (synthetic ALPE)

1-Oleoyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine (750 mg) was dissolved in dry chloroform (25 mL) and triethylamine (270 µL) was added under inert atmosphere. The solution was cooled on an ice bath and linoleic anhydride (930 mg, 1.1 eq.) was added dropwise with stirring. The solution was left overnight at room temperature (nitrogen atmosphere) and then concentrated to give a crude oil, which was purified by flash chromatography ($CHCl_3$/MeOH/H_2O) to give the pure product *N*-linoleoyl-1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamide (0.54 g, 45%). The structure was verified by ^1H NMR ($CDCl_3$/CD_3OD): 5.30 ppm (m, 6H, 3 x CH=CH), 3.6-3.8 ppm (m, 7H, *sn*-1,2,3, CH_2OPO), 3.12 ppm (t, 2H, CH_2N), 2.74 ppm (t, 2H, =CH$\underline{CH_2}$CH=), 2.32 ppm (t, 2H, CH_2COO), 2.18 ppm (t, 2H, CH_2CONH), 2.00 ppm (m, 8H, $\underline{CH_2}$CH=), 1.60 ppm (m, 4H, $\underline{CH_2}CH_2CH$=), 1.30 ppm (m, 18 x CH_2), 0.89 ppm (m, 6H, 2 x CH_3).

An impure fraction (0.82 g) containing the product (~30%) and linoleic acid was collected for further purification.

Example 2: Screening of lipolytic enzymes by ALPE plate assay

Preparation of ALPE plates

ALPE isolated from wheat flour was used to prepare plates for assay as follows:

A) 50 ml 2% agarose in purified water was melted/stirred in a microwave oven and cooled to 60 °C.

B) 20 ml 2% ALPE in 0,2M NaOAc, 10 mM $CaCl_2$, pH 5,5 was kept at 60°C for 10 min. and was blended for 30 sec. with ultrathorax.

Equal volumes of A) and B) were mixed, 100 µl 4 mg/ml crystal violet in purified water was added as indicator. The mixture was poured into appropriate petri dishes (e.g. 40ml in a 14cm Ø dish or 20 ml in a 9 cm Ø dish), and appropriate holes were made in the agar (3-5 mm) for application of enzyme solution.

Screening of lipolytic enzymes

A number of lipolytic enzymes were prepared in isolated form. The enzyme samples were diluted to a concentration corresponding to OD_{280} = 0.5 and 10 microliter was applied into holes in the agarose/ ALPE -matrix. Plates were incubated at 30°C and clearing zones in the plates were identified after incubation for 20 hours . The results were expressed on a semi-quantitative scale from A (largest clearing zone) to E (virtually no clearing zone).

Screening results

The *Fusarium oxysporum* lipase was chosen as a control, and a larger clearing zone than the control was observed for two of the 20 lipolytic enzymes tested.

Example 3: TLC assay

A lipolytic enzyme is incubated with 2% ALPE/APE (0.1M Tris-HCl, pH7.0) at 32 degree, 1350 rpm for 4h. After the reaction, eppendorf tubes are moved to ice-bath.

Samples for TLC are taken out and applied onto a Silica gel 60 F_{254} aluminium sheet (Merck). The plate is eluted in chloroform-methanol-water 65:25:4 (v/v/v) and dried in air (fume hood). The phospholipids are visualized by dipping the plate in a bath of 10% $CuSO_4$ in 8% H_3PO_4 (fume hood) or alternatively 2 M H_2SO_4. After air-drying, the plate is heated using a heat gun (until spots visualize) or oven (5 min at 200°C).

The exact composition of the eluent is known to most strongly influence the distance of migration so freshly prepared eluents should always be used. Care should be taken that the TLC tank is tightly closed in order to avoid evaporation. The typical Rf values for the reference compounds are not always reproducible, so standards should always be applied onto the plate):

FFA (free fatty acid)	0.80
APE	0.55
ALPE	0.40

Example 4: HPLC test

ALPE

ALPE is dissolved in NaOAc buffer pH 5. 500µl substrate solution is heated for 10min. at 30°C. 50µl enzyme solution is added for a reaction period of 10-180min. After the reaction 100µl sample is inactivated at 95°C for 5min. 900µl chloroform/methanol (1:1) is added to the sample. The total sample is centrifuged and analyzed by HPLC (Microsorb-MV 100Si 250mm column, analytical instruments. Mobile phases: A: 80% $CHCl_3$, 19.5% MeOH, 0.5% NH_4OH; B: 60% $CHCl_3$, 34% MeOH, 0.5% NH_4OH, 5.5% H_2O, running with gradient. Detector: Sedere, Sedex 75 light scattering, Temp 40°C, pressure 3.5 Bar.

APE

APE and N-GPE (e.g. 1:1) are mixed in a NaOAc buffer pH 5 30°C by mixing with an Ultra Thurax. 500µl substrate solution is heated for 10min. at 30°C. 50µl enzyme solution is added for a reaction period of 10-180min. After the reaction 100µl sample is inactivated at 95°C for 5min. 900µl chloroform/methanol (1:1) is added to the sample. The total sample is centrifuged and analyzed by HPLC (Microsorb-MV 100Si 250mm column, analytical instruments. Mobile phases: A: 80% $CHCl_3$, 19.5% MeOH, 0.5% NH_4OH; B: 60% $CHCl_3$, 34% MeOH, 0.5% NH_4OH, 5.5% H_2O, running with gradient. Detector: Sedere, Sedex 75 light scattering, Temp 40°C, pressure 3.5 Bar.

Example 5: Baking

A lipolytic enzyme is selected on the basis of screening with APE or ALPE substrate and optionally other test substrates. The selected enzyme is added to dough at a dosage in range 0.1 - 10 mg enzyme protein per kg flour (e.g. about 1 mg/kg).

Doughs are prepared according to a standard European straight dough procedure with 100 parts (by weight) of flour, 4 parts of yeast, 1.5 parts of salt, and 1.5 parts of sugar and water optimized to the flour. Doughs are scaled for rolls or pan bread. The volume of the bread is measured by the rape seed displacement method and the shape of the rolls is measured as the ratio between height and width.

CLAIMS

1. A method of screening a lipolytic enzyme for use as a baking additive, comprising:
   a) contacting the enzyme with N-acyl phosphatidyl ethanolamine (APE) or N-acyl lysophosphatidyl ethanolamine (ALPE), and
   b) detecting hydrolysis of an ester bond in the APE or ALPE.

2. The method of the preceding claim which further comprises:
   a) contacting the enzyme with phosphatidyl choline (lecithin), and
   b) detecting hydrolysis of an ester bond in the lecithin.

3. The method of either preceding claim which further comprises:
   a) contacting the enzyme with a C_{16}-C_{20} triglyceride, and
   b) detecting hydrolysis of an ester bond in the triglyceride.

4. The method of any preceding claim which further comprises:
   a) contacting the enzyme with a monoglyceride, and
   b) detecting hydrolysis of an ester bond in the monoglyceride.

5. The method of any preceding claim which further comprises:
   a) contacting the enzyme with lysophosphatidyl choline (lysolecithin), and
   b) detecting hydrolysis of an ester bond in the lysolecithin.

6. The method of any preceding claim which further comprises:
   a) contacting the enzyme with digalactosyl diglyceride (DGDG), and
   b) detecting hydrolysis of an ester bond in the DGDG.

7. The method of any preceding claim which subsequently comprises:
   a) adding the enzyme to a dough and baking the dough to make a baked product, and
   b) measuring loaf volume or dough stability of the baked product.